Bull. Environ. Contam. Toxicol. (1994) 53:309–316

Environmental Contamination and Toxicology

ATP Assay on Cell Monolayers as an Index of Cytotoxicity

A. Castaño,[1,2] J. V. Tarazona[1]

[1]Division of Environmental Toxicology, CISA-INIA, E-28130 Valdeolmos, Madrid, Spain
[2]Toxicology Department, I Carlos III, Majadahonda, Madrid, Spain

Received: 15 March 1993/Accepted: 17 January 1994

In vitro testing using cell cultures has proven to be a useful tool to screen for the general toxicity of chemicals (Knox et al. 1986; Babich et al. 1986; Kemp et al. 1986). As a result, a large number of in vitro assays have been proposed in recent years, which include a variety of endpoints in order to show the cytotoxic effects of chemicals. The most common endpoints used are those related to general toxic effects such as cell viability, membrane integrity, changes in macromolecular contents (DNA, RNA, proteins) cell proliferation, or general metabolic effects such as ATP content (Kemp et al. 1985, 1986; Galli et al. 1991; Andersson et al. 1990)

From a biochemical point of view, ATP content is considered as the "pulse" of aerobic cells and is an excellent indicator of cellular health (Kemp et al. 1985) since it is the prime energy donor (Kemp et al. 1986). Thus, ATP estimations can provide reliable information on the physiology of a cell and the effects of toxicants (Block et al. 1989). As early as 1969, ATP content was used as an indicator of cytotoxicity in Ehrlich ascites cells (Nungester et al. 1969). However, since the commercial purification of the luciferin/ luciferase system (De Luca and McElroy 1981), ATP determination by bioluminescence has become an easy, rapid and highly sensitive parameter all of which has increased its use as a cytotoxicity endpoint.

The first step in ATP determination is its extraction from the cell. This can be achieved by acid extraction (Chapin et al. 1988; Guille et al. 1989) or by using a nonionic detergent (Köscegui et al. 1988). Acid extraction, of necessity, lysates cells resulting in the liberation of structural and soluble protein components, while nonionic detergents permeabilize cells permitting ATP liberation that is not immediately followed by the complete release of the intracellular "soluble" components (Köscegui et al. 1988).

ATP determination by bioluminescence, extracted by nonionic detergents was

Present address: Sanidad Animal, INIA, E-28130 Valdeolmos, Madrid, Spain
Correspondence to: A. Castaño

widely used as a cytotoxicity index, when working with cell suspensions (Kemp et al. 1985; 1986; Andersson et al. 1990), but with cell monolayers, cell suspensions are usually obtained first after trypsin treatment (Becerril et al. 1989).

This work presents a protocol to evaluate ATP content as a cytotoxicity index, using direct extraction of cell monolayers by commercial equipment and reagents.

MATERIALS AND METHODS

RTG-2 cells, an established fibroblastic-like cell line (ATCC CCL 55), derived from rainbow trout, were grown in Minimum Essential Medium, Eagle modified with Earle's salts with non-essential amino acids, 200 IU/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL Fungizone, 2mM L-glutamine and supplemented with 10% foetal bovine serum (Flow, Scotland), in 75 cm^2 flasks (Costar) gassed with 5% CO_2 in air, tightly closed and maintained at 20 °C.

Cells in the exponential growth phase were rinsed with phosphate buffered saline (PBS), and dissociated with trypsin-EDTA (Flow, Scotland). Individual wells of a 96-well tissue culture microtitre plate (Costar) were inoculated with 0.2 mL of medium containing different concentrations of cells from 500 to 20,000. The plates were incubated at 20 °C in sealed plastic bags insufflated with a mixture of 5% CO_2 in air for 24 hr to allow cell attachment.

ATP measurements were made directly on the cell monolayers according to the following protocol: medium from each well was removed carefully by suction, and cells were washed twice with PBS, pH=7.3. Immediately, 0.2 mL of the detergent, Nucleotid Releasing Reagent for Somatic cells (NRS, Lumac, The Netherlands), was added to each well. After 30 sec, NRS was recovered into individual polypropylene tubes containing 25 mM N-2-Hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) (Sigma), pH 7.75 or PBS to dilute the sample.

Tubes containing the extracted ATP were maintained on ice and readings did not change after an interval of 3 hr. It is possible to preserve these samples for a week if vials are frozen at -20°C. ATP determinations were performed in a 3M-2010 Biocounter (Lumac, the Netherlands). 200 μL of sample were introduced into a lumacuvette and after the addition of 100 μL of the enzyme Luciferin-luciferase (Lumit, Lumac, The Netherlands) readings were performed within 10 sec. In these conditions one thousand counts was defined as a relative light unit (RLU). A curve of relative light units plotted against ATP concentration was used to convert the measured values into amount of ATP.

Standard curves were performed as follows: 10 μg ATP (Lumac, The Netherlands) were dissolved in sterile, bidistilled water, aliquoted in 0.5 mL vials of 2 μg ATP/mL and stored in liquid nitrogen until their use. One aliquot was diluted in PBS or HEPES 25 mM, pH=7.75, covering a range from 0 to 20 ng ATP.

Cytotoxicity assays: 96 well plates were seeded with 0.2 mL of complete medium containing 15,000 cells/well and incubated as previously described for 48 hr (until the cells reached exponential grown phase). Thereafter, the medium was removed and 0.2 mL of test medium, containing 1% instead of 10% of foetal bovine serum (FBS), with various concentrations of test chemicals, was added to each well with eight replicate wells per concentration. Eight control culture wells, including solvent control when necessary, were located at random on the plate and received the same medium without test chemicals. After incubation for another 48 hr, in the conditions above described, the medium was removed and the ATP assay by the direct extraction method was performed. Two reference chemicals were used in this case, one organic (phenol, Merck) and one inorganic (copper nitrate, Merck). Stock solution of phenol was prepared in ethanol. The test solutions were diluted in test medium (1% FBS) with a final solvent concentration always below 1.5% v/v.

Cytotoxicity of both chemicals was also determined by protein measurement using Kenacid Blue Protein assay (Knox et al. 1986). Protein determinations were performed in four wells per concentration on the same plate. Three plates for each compound were used.

Probit analysis was used to determine the median effective concentration (EC50), that concentration resulting in a 50% decrease in ATP or protein content, for each chemical by means of a computer program (Trevors 1985).

RESULTS AND DISCUSSION

Adequate levels of ATP are essential for a variety of transport, repair and synthetic processes. It is not surprising that chemically induced prolonged depletion of ATP either as a consequence of its impaired synthesis or enhanced destruction can result in toxicity. Changes in the amount of ATP or other intracellular components are characteristic of cells undergoing necrosis (Bridges et al. 1983; Vernetti et al. 1993).

In cytotoxicity tests, this chemically induced depletion causes enormous differences between the ATP content of nontreated (controls) versus treated cells. The range of several orders of magnitude required for these assays, can be obtained with bioluminescence, by using buffers with high osmolarity as has been proposed by Chapin et al.(1988); but this effect implies a loss in sensitivity. As can be seen in Figure 1, when using PBS the slope of the standard curve is lower than that obtained when HEPES 25mM was used as ATP solvent. The other method of covering a wide range of measurements is to perform serial dilutions of the sample. In this case, it is absolutely necessary that the solvent in which the ATP is maintained be able to offer a constant linear relationship.

The use of PBS as a solvent proposed by Galli et al. (1991) gave a loss in linearity when ratios between the extraction detergent (NRS) and solvent (PBS)

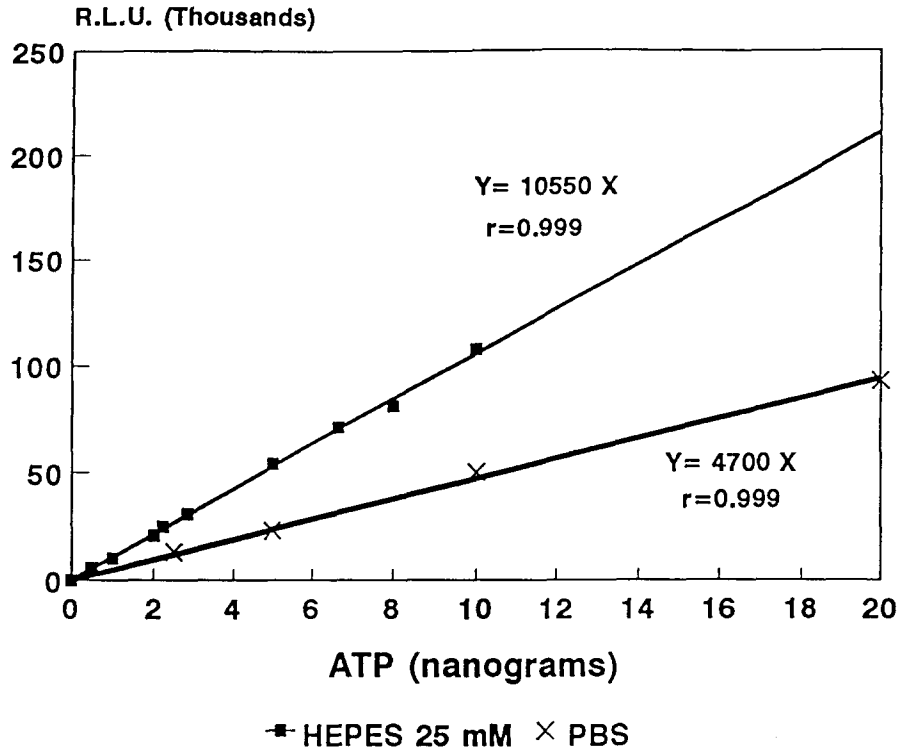

Figure 1. Standard ATP curves using PBS and HEPES 25 mM as solvents.

did not remain constant (Figure 2). By contrast, the use of HEPES 25 mM resulted in good linearity. This buffer has been also proposed by Guille et al (1989) after acid extraction.

In a cytotoxicity test the effect of chemicals upon the plasma membrane could involve severe damage to it, such that part of the intracellular ATP may be released from the cell (Andersson et al. 1990). Trypsin could accentuate membrane damage differentially resulting in errors in the quantitative estimation of total intracellular ATP. For this reason, in addition to the manipulation and time required by the indirect protocol, the use of trypsin to obtain cell suspensions could involve serious difficulties.

The protocol proposed here provides an extremely simple method of estimating the ATP content of cells which circumvents the need for trypsinization. The use of this system of serial dilutions, allows us to work with a method much more sensitive than those used by other authors (Chapin et al. 1988; Galli et al. 1991). In fact, with the direct extracting protocol proposed here, the lower limit of detection is about 500 cells/well (Figure 3) and the upper limit of detection can be as high as needed because the sample can be diluted.

When the proposed protocol was applied to these two reference chemicals (Figures

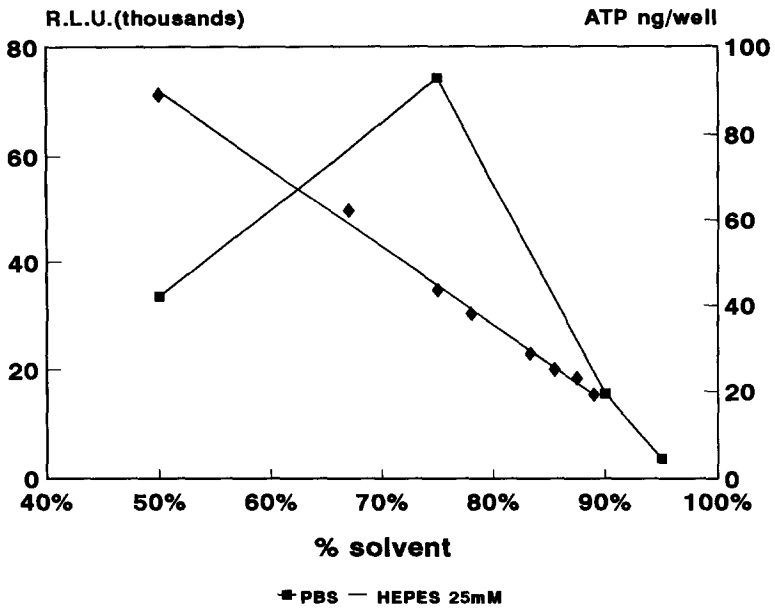

Figure 2. The effects of the PBS and Hepes 25 mM on the linearity of the dilutions of the extracted ATP.

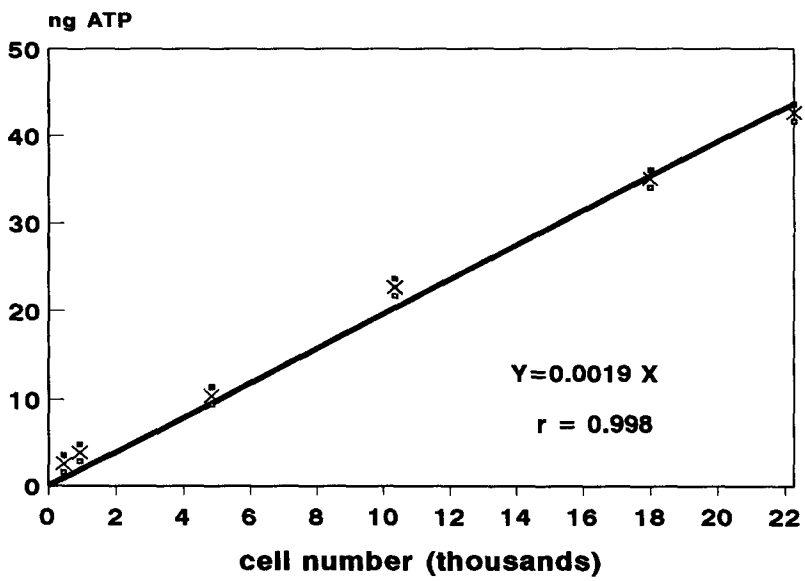

Figure 3. Relationships between ATP content versus cell number.

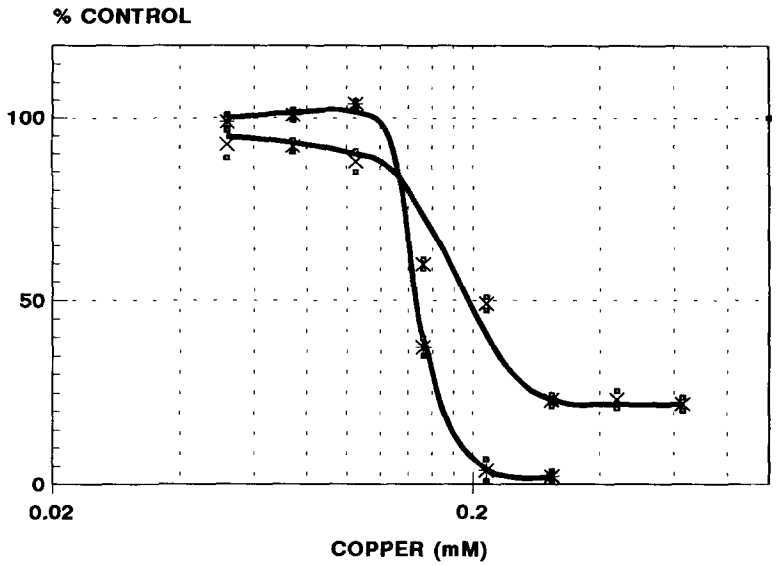

Figure 4. Cytotoxicity curves of copper using ATP direct extraction and protein content as endpoints. Cytotoxicity was evaluated as the reduction in ATP and protein content, and expresed as a percentage of the control values. SEM = standard error of the media.

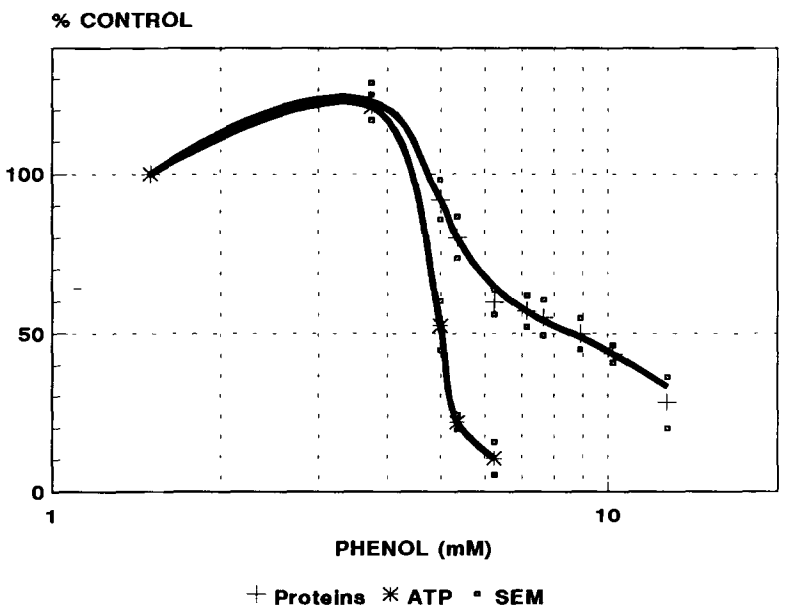

Figure 5. Cytotoxicity curves of phenol using ATP direct extraction and protein content as endpoints. Cytotoxicity was evaluated as the reduction in ATP and protein content, and expresed as a percentage of the control values. SEM = standard error of the media.

4 and 5), the shape of both dose-response curves shows that the use of ATP levels as a cytotoxicity index seems to be a more sensitive parameter of cytotoxicity than protein estimation. In fact, the EC50 for ATP (Table 1) is lower in both chemicals than the EC50 obtained for proteins. The EC50 for ATP is also lower for both chemicals than the EC50 reported in the same cells when other cytotoxicity endpoints such as viability (Babich et al. 1987) or cell attachment were employed (Bols et al. 1985).

Correlation coefficients (r) for log-Probit fittings were 0.94 and 0.96 for ATP and proteins of copper and 0.95 and 0.94 for ATP and proteins of phenol.

Table 1. EC50 (mM) and its 95% confidence levels of copper and phenol determined by ATP content and the protein assay.

	EC50 ATP	95% level	n	EC50 Protein	n	95%level
copper	0.148	0.140-0.156	12	0.19	18	0.17-0.21
phenol	4.99	4.88-5.11	12	8.65	24	8.17-9.16

Low concentrations of phenol showed values slightly higher than controls. Although this could be considered a positive response of cell growth to low concentrations of phenol, this fact does not affect to the calculation of the EC50 values.

The high sensitivity and the absence of artifacts, its versatility, speed and simplicity lead us to recommend this protocol in routine cytotoxicity assays. The same protocol can be used with almost all cell types grown on monolayers if both the seeding concentration of the cells and the time interval chosen for toxic exposition are taken into account and directly related to the size and growth cycle kinetics of the cells. These must be modified according to the cell type, especially in homoiothermic species.

Acknowledgments. We thank M. Carballo for her help. This work was partially supported by CEE research project AIR 1-ZT92-0036.

REFERENCES

Andersson M, Forsby A, Lewan L (1990) Determination of ATP leakage from cultured cells in toxicity testing: a two step bioluminiscent assay. ATLA 17:188-190

Babich H, Shopsis C, Borenfreund E (1986) In vitro Cytotoxicity testing of aquatic pollutants (cadmium, copper, zinc, nickel) using established fish cell lines. Ecotoxicol Environ Saf 11:91-99

Becerril C, Hidalgo R, Santamaria A, Sanz F (1989) Determinación de ATP en

células V79 y CHO. 1st Meeting Soc. Esp. Mutag. Ambient. Barcelona (Spain)
Bols NC, Boliska SA, Dixon DG, Hodson PV, Kaiser KLE (1985) The use of fish cell cultures as an indication of contaminant toxicity to fish. Aquat Toxicol 6:147-155
Block JC, Bauda P, Reteuna C (1989) Ecotoxicity testing using aquatic bacteria. In: Boudou A & Ribeyre F (eds) Aquatic Ecotoxicology Fundamental Concepts and Methodologies, vol 2. CRC Press, Boca Raton, p 187
Bridges JW, Benford DJ, Hubbard SA (1983) Mechanisms of toxic injury. Ann NY Acad Sci, vol 407, p 42
Chapin RE, Gray TJB, Phelps JL, Dutton SL (1988) The effects of mono-(2-ethylhexyl)-phthalate on rat sertoli cell-enriched primary cultures. Toxicol Appl Pharmacol 92:467-479
De Luca MA, McElroy WD (1981) Bioluminiscence and Chemiluminiscence. Academic Press, New York, p 122
Galli CL, Viviani B, Casartelli G, Marinovich M (1991) ATP and protein synthesis assays to evaluate the toxicities of preservatives in vitro. ATLA 19:60-67
Gille JJP, Berkel CGM, Mullaart E, Vijg J, Joenje H (1989) Effects of lethal exposure to hyperoxia and to hydrogen peroxide on NAD(H) and ATP pools in chinese hamster ovary cells. Mutat Res 214:89-96
Kemp RB, Cross DM, Meredith RWJ (1986) Adenosine triphosphate as an indicator of cellular toxicity in vitro. Fd Chem Toxic 24:465-466
Kemp RB, Meredith RWJ, Gamble SH (1985) Toxicity of commercial products on cells in suspension culture: A possible screen for the draize eye irritation test. Fd Chem Toxic 23:267-270
Knox P, Uphill PF, Fry JR, Benford J, Balls M (1986) The frame multicentre project on in vitro cytotoxicology. Fd Chem Toxic 24:457-463
Köszegi T, Kellermayer M, Kövecs F, Jobst K (1988) Bioluminescent monitoring of ATP release from human red blood cells (treated with nonionic detergent). J Clin Chem Clin Biochem 26:599-604
Nungester WJ, Paradise LJ, Adair JA (1969) Loss of cellular ATP as a measure of cytolytic activity of antiserum Proc Soc Exp Biol Med 132:582
Trevors JT (1986) A basic program for estimating LD50 values using the IBM-PC. Bull Environ Contam Toxicol 37:18-26
Vernetti LA, MacDonald JR, Wolfgang GHI, Dominick MA, Pegg DG (1993) ATP depletion is associated with cytotoxicity of novel lipid regulator in guinea pig adrenocortica cells. Toxicol Appl Pharmacol 118:30-38